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Capturing genes encoding membrane and secreted proteins important for mouse development

(mouse embryonic stem cells/gene trap/insertional mutation)

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ABSTRACT **A strategy based on the gene trap was developed to prescreen mouse embryonic stem cells for insertional mutations in genes encoding secreted and membrane-spanning proteins. The "secretory trap" relies on capturing the N-terminal signal sequence of an endogenous gene to generate an active β-galactosidase fusion protein. Insertions were found in a cadherin gene, an *unc6*-related laminin (netrin) gene, the *sek* receptor tyrosine kinase gene, and genes encoding two receptor-linked protein-tyrosine phosphatases, LAR and PTPκ. Analysis of homozygous mice carrying insertions in LAR and PTPκ showed that both genes were effectively disrupted, but neither was essential for normal embryonic development.**

The identification and mutation of secreted and transmembrane proteins expressed during early mouse embryogenesis is a prerequisite for understanding cell–cell interactions required for mammalian development. Expression cloning methods to isolate embryonic cDNAs encoding this class of proteins (1, 2) are technically demanding and potentially biased in favor of smaller, abundantly transcribed mRNAs. Gene trapping in mouse embryonic stem (ES) cells offers a rapid, but essentially random, method to identify and simultaneously mutate genes expressed during mouse development (3). Because gene trapping relies on random insertion into the genome of cells, the detection of genes should not be influenced by the relative abundance of transcripts in ES cells. However, it is anticipated that genes composed of large introns will be more readily detected by gene trap vectors as they present a larger target for insertion.

Conventional gene trap vectors contain a splice acceptor sequence linked to the *lacZ* or βgeo reporter gene (4–8); the latter is a *lacZ*–neomycin phosphotransferase fusion gene. When these vectors integrate within the introns of genes, β-galactosidase (β-gal) fusion proteins are produced that include the N terminus of the endogenous gene present at the site of insertion. β-gal enzyme activity in cell lines transfected with gene trap constructs has been observed in a variety of subcellular locations (8, 9), presumably reflecting the acquisition of endogenous protein domains that act to sort the fusion protein to different intracellular compartments. Here, we have exploited the differential sorting of β-gal fusion proteins as a means to capture genes encoding N-terminal signal sequences, genes therefore likely to be expressed on the cell surface.

MATERIALS AND METHODS

Vectors. The βgeo reporter in all vectors was obtained from pGT1.8geo by replacing the *Cla* I (unique in *lacZ*)/*Sph* I (unique in *neo*) fragment of the gene trap vector pGT1.8 with the *Cla* I/*Sph* I fragment of pSAβgeo (7). pGT1.8 is a derivative of pGT4.5 (4) where the 3′ *En2* sequences were replaced with the 0.2-kb *Bcl* I/*Bam*HI simian virus 40 poly(A) signal (10). The parental vector pActβgeo contains the 0.5-kb human β-actin promoter (10) linked to the βgeo/simian virus 40 poly(A) cassette. The start of βgeo translation was engineered to contain a Kozak consensus sequence with unique *Sal* I and *Nru* I sites on either side for generating subsequent fusions (SDK oligonucleotide: 5′-GTCGACCTGCAGGTCGGAGGCCACCATGGCTCGCGAT, from S. Darling, Medical Research Council Mammalian Development Unit, London). *Sal* I sites were placed at each end of a *Bal* I fragment containing the entire coding region of the rat CD4 cDNA (11). A 0.45-kb *Sal* I/*Kpn* I fragment containing the N-terminal cleavable signal sequence (SS) of CD4 or a 1.4-kb *Sal* I/*Nde* I fragment containing the entire CD4 coding region was cloned into *Sal* I/*Nru* I-digested pActβgeo to generate pActSSβgeo and pActSSTMβgeo, respectively. The secretory trap vector pGT1.8TM includes the 0.7-kb *Pst* I/*Nde* I fragment of CD4 containing the transmembrane domain (TM) inserted in-frame with βgeo in pGT1.8geo.

ES Cell Culture. CGR8 ES cells (a feeder-independent cell line derived from strain 129/Ola mice by J. Nichols; ref. 12) were maintained in Glasgow MEM/BHK12 medium containing 0.23% sodium bicarbonate, 1× MEM essential amino acids, 2 mM glutamine, 1 mM pyruvate, 50 μM 2-mercaptoethanol, 10% (vol/vol) fetal calf serum (Globepharm, Surrey, U.K.), and 100 units of differentiation-inducing activity/leukemia-inhibitory factor per ml. Transiently transfected cells were obtained by electroporating 10^7 ES cells with 100 μg of uncut plasmid DNA in a volume of 0.8 ml of PBS by using a Bio-Rad Gene Pulser set at 250 μF/250 V and cultured for 36 hr on gelatinized coverslips prior to analysis. To obtain stable cell lines, between 5×10^7 and 10^8 CGR8 ES cells were mixed with 150 μg of linearized plasmid DNA and electroporated at 3 μF/800 V. Cells (5×10^6) were plated on 10-cm dishes and selected in the presence of Geneticin (GIBCO) at 200 μg/ml. To assay β-gal enzyme activity and protein, ES cells were grown on gelatinized coverslips and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (13) or with polyclonal rabbit anti-β-gal antiserum (a gift from J. Price, National Institute of Medical Research) and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) (14). To permeabilize membranes, cells were treated with 0.5% Nonidet P-40 prior to antibody staining.

RNA Analysis and Rapid Amplification of cDNA Ends (RACE) Cloning. Northern blots and 5′ RACE cloning were

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Abbreviations: ES, embryonic stem; β-gal, β-galactosidase; SS, N-terminal cleavable signal sequence; TM, transmembrane domain; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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carried out as described (8). Probes containing the cytoplasmic region and 3′ untranslated region of the genes encoding the protein-tyrosine phosphatases LAR and PTPκ were generously provided by B. Goldstein (Thomas Jefferson University, Philadelphia) and J. Sap (New York University Medical Center, New York). Several modifications were incorporated into the 5′ RACE procedure: (*i*) microdialysis (0.025-μm filters; Millipore) was used in place of ethanol precipitation, (*ii*) nested PCR (30 cycles each) was carried out using an anchor primer (5′-GGTTGTGAGCTCTTCTAGATGG) and a primer specific to CD4 (5′-AGTAGACTTCTGCACAGACACC) followed by size selection on agarose gels and a second round of PCR with the anchor and the *En-2* 256 (8) primers, and (*iii*) Chromo Spin 400 columns (Clontech) were used to size select *Xba* I/*Kpn*-digested PCR products prior to cloning.

ES Cell Chimeras. Chimeric embryos and germ-line mice were generated by injection of C57BL/6 blastocysts (8). Embryos at the appropriate stages were dissected, fixed, and stained with X-Gal (13).

RESULTS AND DISCUSSION

To test if β-gal fusions that contain an SS could be identified by their subcellular distribution, vectors were constructed to express portions of the CD4 type I membrane protein (11) fused to βgeo, a chimeric protein that possesses both β-gal and neomycin phosphotransferase activities (7) (Fig. 1*A*). βgeo fused to the signal sequence of CD4 (pActSSβgeo) accumulated in the endoplasmic reticulum (ER) but lacked β-gal activity (Fig. 2 *C* and *D*). Therefore, translocation of βgeo into the lumen of the ER appeared to abolish β-gal enzyme function. β-gal activity was restored by including the TM of CD4 (pActSSTMβgeo) (Fig. 2 *E* and *F*), presumably by keeping β-gal in the cytosol. Active protein was localized in the ER and in multiple cytoplasmic inclusions, a pattern only rarely observed in ES colonies obtained with the conventional gene trap vector, probably because insertions downstream of *both* a signal sequence and TM of genes encoding membrane-spanning proteins are infrequent. Therefore, to identify insertions in both secreted and type I membrane proteins, our gene trap vector pGT1.8geo was modified to include the TM of CD4 upstream of βgeo (Fig. 1*B*). With the secretory trap vector pGT1.8TM we would now expect to restore β-gal enzyme activity to any insertion occurring downstream of a signal sequence.

In a pilot experiment, the relative efficiency of our gene trap vector was compared to the original pSAβgeo (7) after electroporation into ES cells (Fig. 1*B*). Although pSAβgeo contains a start of translation that is absent in our vectors, fewer G418-resistant colonies were obtained with pSAβgeo than with pGT1.8geo. More importantly, nearly all the colonies derived with pSAβgeo showed high levels of β-gal activity, whereas our vector showed a broad range of staining intensities and a greater proportion of β-gal-negative colonies. Sequence analysis of the pSAβgeo vector revealed a point mutation in *neo* known to reduce its enzyme activity (15). Therefore, the

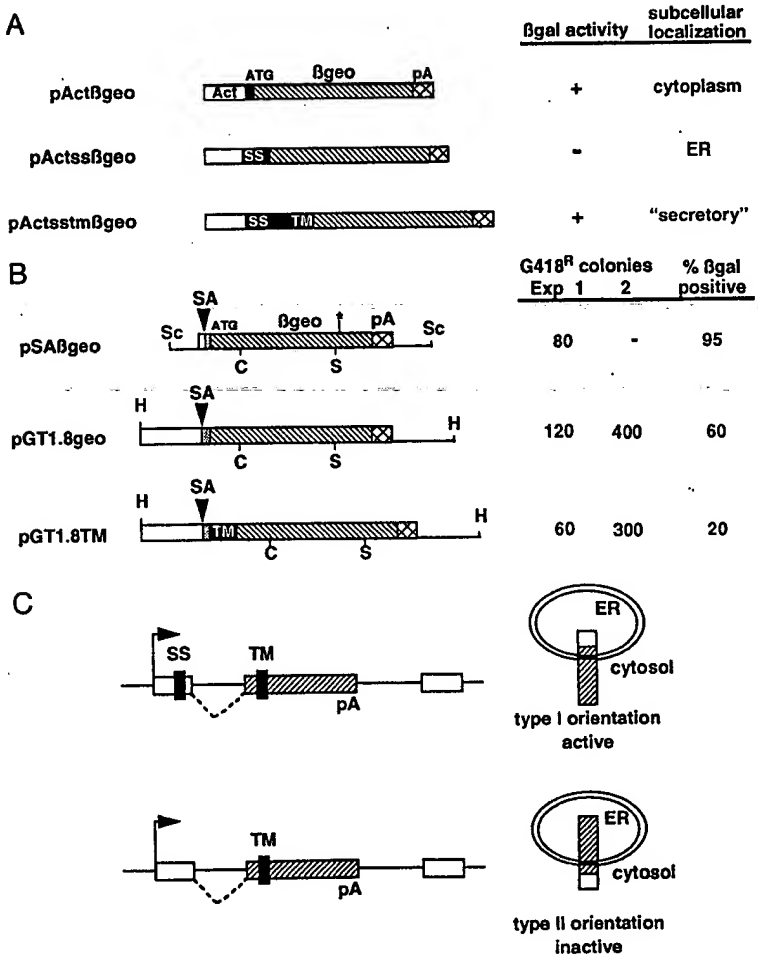

FIG. 1. The secretory trap vector shows selective activation of the β-gal enzyme in fusions that capture an SS. (*A*) CD4–βgeo expression constructs. The human β-actin (Act) promoter was used to drive expression of βgeo alone (pActβgeo), fused in frame with the SS of CD4 (pActSSβgeo), or fused to the SS and TM of CD4 (pActSSTMβgeo). Results of transient transfection experiments shown in Fig. 2 are summarized on the right. (*B*) Relative efficiency of gene trap and secretory trap vectors in ES cells. pSAβgeo (7) contains the minimal adenovirus type 2 major late splice acceptor (SA, arrowhead; open box, intron; shaded box, exon) and the bovine growth hormone polyadenylylation signal (pA). The mutation in *neo* (*) present in pSAβgeo was corrected in our vectors by replacement of the *Cla* I (C)/*Sph* I (S) fragment of βgeo. pGT1.8geo and pGT1.8TM contain the mouse *En-2* splice acceptor and simian virus 40 polyadenylylation signal and lack a translation initiation signal (ATG). Vectors were linearized prior to electroporation at either the *Sca* I (Sc) site of the plasmid backbone (represented by the line) of pSAβgeo or at the *Hin*dIII (H) site at the 5′ end of the *En-2* intron. The number of G418-resistant ($G418^R$) colonies obtained in the electroporation of 5×10^7 (experiment 1) or 10^8 (experiment 2) cells and the proportion that express detectable β-gal activity are indicated on the right. (*C*) Model for the selective activation of β-gal in the secretory trap vector. Insertion of pGT1.8TM in genes that contain an SS produces βgeo fusion proteins that are inserted in the membrane of the endoplasmic reticulum in a type I configuration. The TM of the vector retains βgeo in the cytosol where β-gal remains active. Insertion of the vector in genes that lack a signal sequence produces fusion proteins with an internal TM domain. Insertion of these proteins in a type II orientation exposes βgeo to the lumen of the ER where β-gal activity is lost.

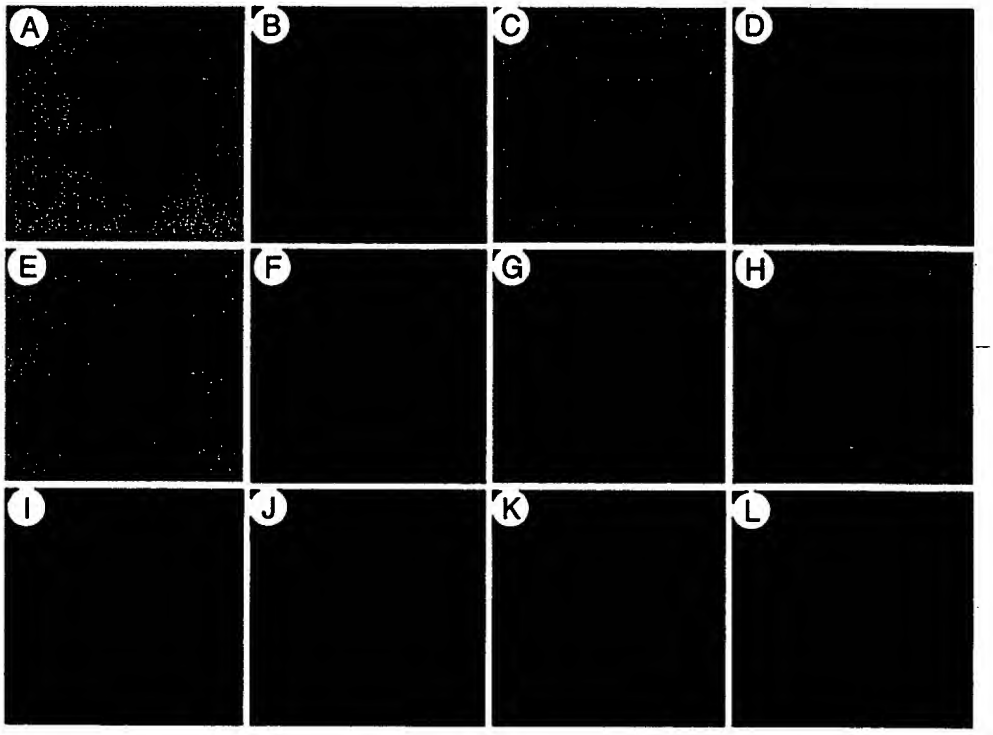

FIG. 2. Localization of βgeo fusion protein and enzyme activity in ES cells. (*A–F*) Transient transfection of ES cells with pActβgeo (*A* and *B*), pActSSβgeo (*C* and *D*), and pActSSTMβgeo (*E* and *F*) and assay for β-gal activity (X-Gal; bright field) and protein (immunofluorescence; dark field). βgeo alone was evenly distributed in the cytoplasm of cells. βgeo fused to the SS of CD4 accumulated in the ER, resulting in the loss of β-gal activity. β-gal activity was restored in fusions that contain both the SS and TM of CD4, and βgeo localizes to the ER and in multiple cytoplasmic inclusions. (*G* and *H*) Stable cell lines transfected with pGT1.8TM showing constitutive β-gal activity (*G*) or activity induced in a subset of differentiated cell types (*H*) in cultures of ST534 and ST519 cells, respectively (*I–L*). Detection of βgeo protein in β-gal-positive (*I* and *J*) and β-gal-negative (*K* and *L*) cell lines in permeabilized (*I* and *K*) and nonpermabilized (*J* and *L*) cells. In β-gal-negative cells, the βgeo fusion was detected on the surface of β-gal-negative cells in the absence of membrane permeabilization, indicating that the loss of β-gal activity correlated with a type II orientation of the fusion protein (see the model in Fig. 1*C*).

pSAβgeo vector appears to preselect for genes expressed at high levels, and correction of the *neo* mutation in our vectors now allows access to genes expressed at low levels (see below).

Approximately half of the pGT1.8geo colonies express detectable β-gal activity and show the various subcellular patterns of β-gal staining observed previously. In contrast, only 20% of the pGT1.8TM colonies express β-gal activity (Fig. 1*B*), and all display the "secretory" pattern of βgeo activity characteristic of the pActSSTMβgeo fusion (compare Fig. 2*E* with Fig. 2 *G* and *H*). The reduction in the proportion of β-gal-positive colonies and the singular pattern of β-gal staining observed with the secretory trap vector suggested that β-gal, but not neomycin phosphotransferase activity, is lost in fusions with proteins that do not possess a signal sequence. Loss of β-gal activity would be predicted to occur if fusions that lack a SS were inserted into the membrane in a type II orientation placing βgeo in the lumen of the ER. To confirm this, several β-gal-negative cell lines were isolated and analyzed by immunofluorescence. In these lines, the fusion protein was detected on the surface of cells in the absence of detergent permeabilization (Fig. 2 *K* and *L*), indicating a type II orientation of the βgeo fusion protein. In contrast, detergent permeabilization was essential to detect the fusion protein in β-gal-positive cell lines (Fig. 2 *I* and *J*), as would be expected for type I membrane proteins. From these data, we propose a model to explain the selective activation of β-gal in pGT1.8TM (Fig. 1*C*). In the absence of a SS, the TM of the fusion protein acts as a signal anchor sequence (16) to place βgeo in a type II orientation, exposing βgeo to the lumen of the ER where β-gal activity is lost. In fusions that contain a SS, the TM in the vector acts to prevent βgeo from entering the ER lumen, thereby preserving its cytosolic enzyme activity.

5′ RACE (17) was used to clone a portion of the endogenous gene associated with secretory trap insertions that express detectable β-gal activity (Table 1). Northern and RNA dot blot analysis showed that approximately one-half (5 of 11 analyzed in this study) of the G418-resistant cell lines fail to properly utilize the splice acceptor and produce fusion transcripts that hybridize within intron sequences of the vector (data not shown). These insertions presumably do not represent true gene trap events and thus were not analyzed further. Northern blot analysis of six properly spliced lines (ST484, ST497, ST514, ST519, ST531, and ST534) detected a unique-sized βgeo fusion transcript in each cell line (Fig. 3*A*). At least two independent RACE cDNAs were cloned from each cell line. The cDNAs obtained from all cell lines except ST514 detected both the fusion transcript and an endogenous transcript common to all cell lines as shown for the ST534 probe (Fig. 3*A*). The ST514 insertion illustrates that genes expressed at very low levels in ES cells can be trapped. In ST514 cultures, β-gal activity was observed only in a few differentiated cells, and accordingly neither the fusion nor the endogenous transcripts could be detected on Northern blots (Fig. 3*A* and data not shown).

Sequence analysis of the RACE cDNAs in all cases showed the proper use of the splice acceptor and a single open reading frame in-frame with βgeo. One insertion occurred in netrin, a gene homologous to the *unc-6* gene of *Caenorhabditis elegans* (18) recently cloned in the chicken (19). The remaining five

Table 1. Identification of the endogenous gene associated with six secretory trap insertions

Cell line	β-gal expression*		Transcript size,† kb		Gene‡	Phenotype§	(wt:het:hom)
	ES	Diff.	Fusion	Endogenous			
484	+	+	7.5	7.5	LAR (1833)	NA	
497	+	+/−	6.5	7	*sek* (1376)	?	
514	−	+/−	ND	ND	Netrin (4721)	NA	
519	−	+/−	>12	>12	Novel cadherin	NA	
531	−	+/−	6.1	5.3	PTPκ (2000)	Viable	(36:57:27)
534	+	+	6.0	7.5	LAR (706)	Viable	(36:79:25)

*Based on X-Gal staining of ES cell cultures that contain a subset of spontaneously differentiated (Diff.) cell types. +/− indicates expression in a subset of differentiated cell types.
†Transcript sizes were determined from Northern blots (Fig. 3 and data not shown). ND, not detected.
‡Numbers in parentheses indicate the insertion site within the endogenous gene based on the nucleotide sequence of rat LAR (GenBank accession no. L11586), mouse *sek* (S51422), chicken netrin 1 (L34549), and mouse PTPκ (L10106). The GenBank accession numbers for the mouse netrin and cadherin genes are U23505 and U23536, respectively.
§Based on the recovery of homozygous (hom) animals at weaning age in litters from heterozygous (het) intercrosses. ?, Phenotype unknown, breeding in progress. NA, not applicable, insertion not in germ line. wt, Wild type.

insertions interrupted the extracellular domains of genes encoding membrane-spanning proteins: a cadherin most closely related to the protein encoded by fat tumor suppressor gene of *Drosophila* (20), the *sek*-encoded receptor tyrosine kinase (21), the receptor-linked protein-tyrosine phosphatase PTPκ (22), and two independent insertions in a second receptor-linked protein-tyrosine phosphatase LAR (23). These results support the prediction that β-gal activity is dependent on acquiring a SS from the endogenous gene at the site of insertion.

Reporter gene activity associated with each insertion was analyzed in embryos (Fig. 4). The pattern of β-gal expression in embryos derived from insertions in the *sek* (ST497) and netrin (ST514) genes was very similar to published RNA *in situ* results for the mouse *sek* (24) and chicken netrin (25) genes, which provides further evidence that gene trap vectors accurately report the pattern of endogenous gene expression (8). Both insertions in LAR (ST484 and ST534) exhibited weak, widespread expression in 8.5-day embryos. The insertion in PTPκ (ST531) showed β-gal expression in endoderm and paraxial mesoderm; the highest expression was observed in newly condensing somites. β-gal expression in tissues of adult mice carrying insertions in LAR and PTPκ correlated well with known sites of mRNA expression (22, 26). The highest levels of β-gal activity were found in the lung, mammary gland, and brain of ST534 (LAR) mice and in the kidney, brain, and liver of ST531 (PTPκ) mice (data not shown).

ES cell lines containing insertions in the LAR, PTPκ, and *sek* genes have been transmitted to the germ line of mice. Thus far, breeding analysis showed that mice homozygous for the LAR and PTPκ insertions are viable and fertile. To confirm that the LAR and PTPκ genes were effectively disrupted, Northern blots of RNA from wild-type and homozygous adult tissues were probed with cDNAs from regions downstream of each insertion site. For both mutations, full-length transcripts were not detected in homozygous animals (Fig. 3*B*). Because secretory trap insertions generate fusions that in some cases will contain a large portion of the extracellular domain of the target gene, the production of both loss of function and gain of function (i.e., dominant-negative) mutations is possible. However, since the βgeo fusions with LAR and PTPκ include <300 amino acids of the extracellular domains of these proteins, these insertions likely represent null mutations. LAR and PTPκ are members of an ever-increasing family of receptor protein-tyrosine phosphatase gene (27). Therefore, the absence of overt phenotypes in LAR and PTPκ mutant mice is likely due to functional overlap between gene family members as has been observed with targeted mutations in multiple members of the myogenic and Src family genes (28–30).

In summary, we have modified the gene trap vector to detect insertions in genes that code for cell surface proteins, a class of genes previously missed by conventional gene trap vector. The strategy relies on the retention of β-gal enzyme activity in reporter gene fusions that acquire a SS (Fig. 1*C*), thus providing a simple selective assay to recover insertional mutations in secreted and type I membrane proteins. The vector was demonstrated to detect genes expressed at very low levels in ES cells; therefore, it should be possible to develop screens to identify lineage-specific genes induced upon ES cell differentiation. A screen for cell surface genes expressed in restricted patterns in the early embryo should help to identify key regulators of cell–cell interactions required for the establishment of patterning of the primary embryonic germ layers.

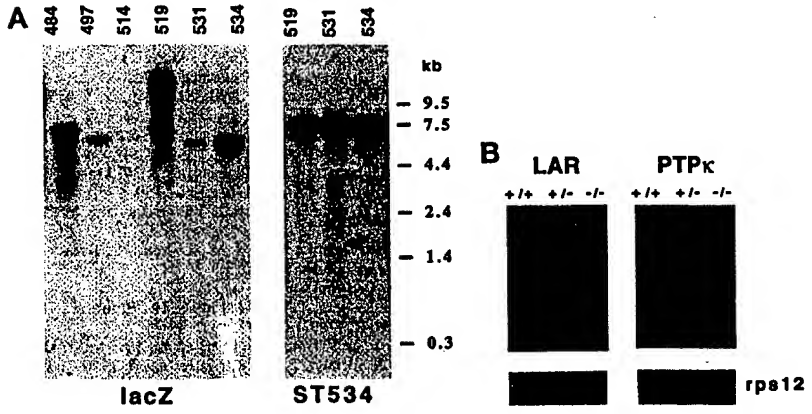

FIG. 3. RNA analysis of secretory trap insertions. (*A*) Northern blot of 15 μg of ES cell RNA hybridized with the *lacZ* gene and reprobed with a RACE cDNA fragment cloned from the ST534 (LAR) insertion. The pGT1.8TM vector is predicted to contribute 5 kb to the size of the fusion transcript. As expected, the ST534 RACE clone detects both the 6-kb fusion transcript in ST534 cells and the 7.5-kb endogenous LAR transcript in all cell lines. (*B*) Northern blots of 10 μg of RNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) lung of ST534 (LAR) and kidney of ST531 (PTPκ) adult mice hybridized with LAR and PTPκ cDNA sequences 3′ to the insertion and reprobed with the ribosomal S12 gene (rps12) as a loading control.

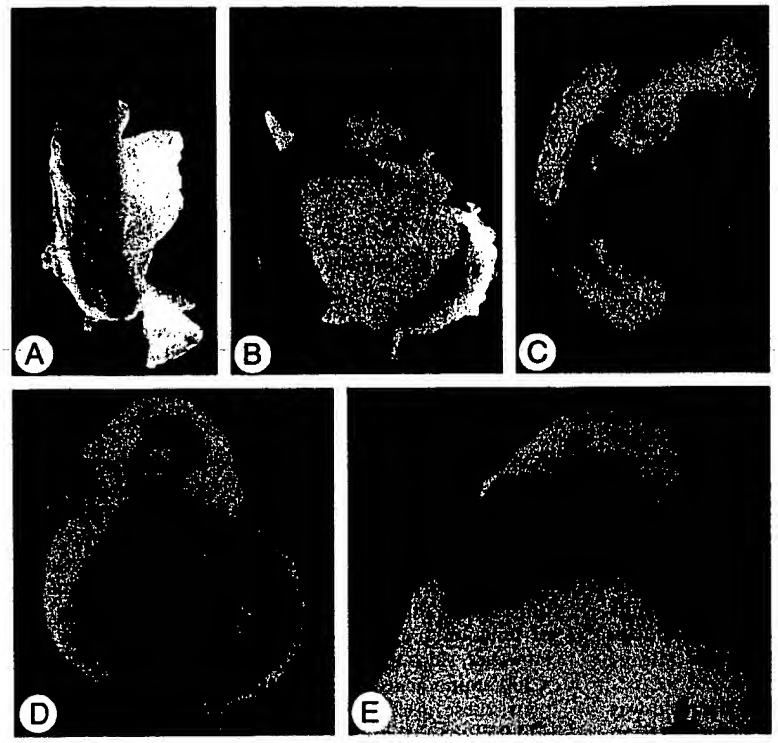

FIG. 4. Expression of the βgeo reporter in embryos carrying insertions of the secretory trap vector. (*A–C*) Heterozygous 9.5-day embryo. (*A*) ST534 (LAR): dorsal view showing widespread, low-level β-gal expression. (*B*) ST531 (PTPκ): lateral view showing high expression in paraxial mesoderm and somites. (*C*) ST497 (*sek*): lateral view showing characteristic *sek* expression in the forebrain, rhombomeres 3 and 5 of the hindbrain, lateral and paraxial mesoderm, and somites (24). (*D* and *E*) ST514 chimeric 10.5-day embryo. (*D*) Lateral view. (*E*) Cross-section showing expression in ventral spinal cord, floorplate, and dermamyotome of somites similar to the pattern described for the chicken netrin genes (25). The arrowhead indicates floorplate, and the arrow indicates dermamyotome.

Furthermore, this approach should be generally applicable to other cultured cell lines.

Based on the first six genes identified, the secretory trap shows a preference for large membrane-spanning receptors. The recovery of two independent insertions in LAR further suggests that the current vector design will access a restricted class of genes. The requirement for gene trap vectors to insert in introns of genes is predicted to impose an inherent bias in favor of detecting genes composed of large intronic regions and consequently limit the number of genes accessible with this approach. To access a larger pool of genes, we have constructed vectors in each of the three possible reading frames. Furthermore, to recover insertions in smaller transcription units composed of few or no introns, we developed an "exon trap" version of the vector that lacks a splice acceptor. Each vector yielded similar numbers of G418-resistant colonies, a similar proportion of which exhibit the secretory pattern of β-gal activity (W.C.S. and J. Brennan, unpublished results). With a combination of vectors, we now expect to obtain a more representative sampling of the genome that should include both membrane receptors and secreted ligands.

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